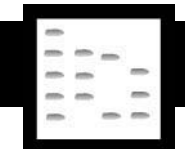
PHEROMONES



Gas Chromatography

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Organisms are able to communicate with each other by means of signal chemicals, or semiochemicals. These are chemicals mediating interactions between individuals, either within the same species (pheromones) or from different species (allelochemicals). Semiochemicals have been most extensively studied for insects, particularly social insects and insects of economic importance. The study of semiochemicals, and the interactions they mediate, is part of chemical ecology and contributes to the understanding of behaviour, development and evolution of organisms.

Semiochemicals are classified according to the relationships between the organisms involved. Pheromones are secreted and released by an organism and cause a specific response in a receiving organism of the same species, whilst allelochemicals are produced by one species and cause a response in a different species. Allelochemicals are further subdivided into allomones, kairomones, synomones and apneumones. Allomones elicit a response that is favourable to the emitter (e.g. defensive secretions utilized against enemies), kairomones favour the receiver (e.g. chemicals that attract parasites or predators), synomones are beneficial to both the emitter and receiver (e.g. floral scents that attract pollinators) and apneumones arise from a nonliving source (e.g. rotting meat odours).

In insects, pheromones are usually produced in specialized glands and perceived by sense organs on the antenna. Their existence has been known since early times. In 1914, the eminent French naturalist and entomologist Fabre reported that a single female of the emperor moth, *Saturnia pavonia*, kept in a wire cage on a window sill, was able to attract a great number of males from kilometres away. However, the first identification of a pheromone was only achieved in 1959. The work was started in 1939 and required the isolation of tens of thousands of excised pheromone glands before the pheromone structure of bombykol [(*E*,*Z*)-10,12-hexadecadien-1-ol] was finally identified. Since then, improvements in isolation and identification techniques have made pheromone identification a simpler and faster procedure, utilizing only a few insects. A large number of pheromones have now been identified for organisms ranging from algae to primates.

Most pheromones fall into three main groups: sex pheromones that influence mate location and courtship behaviour; alarm pheromones that warn neighbours of impending danger; pheromones that influence spacing patterns, e.g. aggregation pheromones. Some pheromones derive specificity from their molecular structure. However, many contain compounds that are common to pheromones from different species or are components of other semiochemical systems. In these cases, specificity can be achieved by employing mixtures having unique relative proportions, with spatial or temporal separation of the organisms often adding to specificity. Although it was thought initially that pheromones would be single components, it is now clear that most are multicomponent systems and that, in many cases, behavioural effects are not mediated by the pheromone alone but require the presence of other semiochemicals. Thus, aggregation of bark beetles on a suitable host tree is mediated by a complex of semiochemicals derived from conspecifics attacking the tree, and from secondary metabolites released by the tree itself.

Structures as simple as ethanol can be employed as pheromone components. However, within the constraints required for aerial transport, considerable specificity can be achieved within one empirical formula by a combination of structural isomerism and stereoisomerism, encompassing both optical and geometrical isomerism. Thus, for the lepidopterous sex pheromones, where the basic structures usually comprise only C_{12}–C_{16} straight carbon chains with functionalities of alcohol, aldehyde and acetate, considerable diversity is obtained by a combination of the position, degree and geometrical isomerism of unsaturated double bonds.

Insect pheromones can employ a range of structures, from fatty acid-based components to polyketides and polyisoprenoids (**Figure 1**). Polyketides can be simple compounds, for example 4-methyl-3,5-heptanedione (Figure 1, structure **1**) the aggregation pheromone of the pea and bean weevil, *Sitona lineatus*, or structures with several chiral centres such

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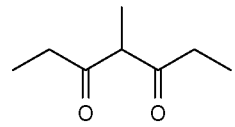

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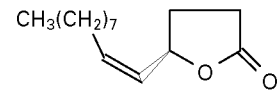
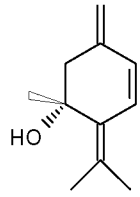
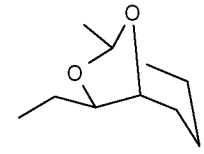


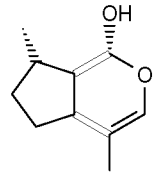
2 (*R,Z*)-5-(1-Decenyl)dihydro-2(3*H*)-furanone



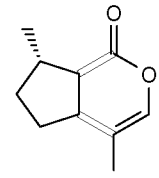
3 Ipsdienol



4 *Exo*-brevicomin



5 (1*R*,4a*S*,7*S*,7a*R*)-Nepetalactol



6 (4a*S*,7*S*,7a*R*)-Nepetalactone

Figure 1 Diversity of structures employed as pheromones. **1** The aggregation pheromone of the pea and bean weevil (*Sitona lineatus*); **2** the sex pheromone of the Japanese beetle (*Popillia japonica*); **3** the aggregation pheromone of the pine engraver (*Ips pini*); **4** the aggregation pheromone of a number of pine beetles; **5** and **6** the sex pheromone of many aphid species.

as *exo*-brevicomin (Figure 1, structure **4**), a component of the aggregation pheromone of a number of bark beetles, including the mountain pine beetle, *Dendroctonus ponderosae*. Simple terpenoids can also be employed, for example (*E*)-citral in the Nasonov pheromone of the honeybee, or highly chiral cyclopentanoid structures comprising the sex pheromones produced by sexual female aphids. For many aphid species, two biosynthetically related compounds are involved, a nepetalactol and a nepetalactone. There are 16 and 8 possible isomers respectively, but only the (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Figure 1, structure **5**) and the (4a*S*,7*S*,7a*R*)-nepetalactone (Figure 1, structure **6**) are behaviourally active.

Isolation Techniques

This is a key stage in the identification of semiochemicals and the techniques used will determine, to a large extent, the subsequent analytical techniques, particularly in terms of sample introduction into the gas chromatograph (GC). Most of the insect pheromones so far identified have been extracted using solvents with a range of polarities and were obtained from whole insects, specific parts of insects (e.g. pheromone glands) or the frass (i.e. the refuse and excrement) produced by feeding insects. In some instances, the pheromone is released from a droplet which can be readily collected. For example, maturing eggs of the mosquito *Culex quinquefasciatus*, a vector of the parasite responsible for the tropical disease filariasis, produce a droplet containing a volatile oviposition pheromone that attracts gravid females of the same species to lay eggs nearby. In this case, sample preparation is a simple matter of collecting the droplets in a fine capillary tube. Extracts made from whole insects or pheromone glands are able, in most cases, to provide a sample containing the compounds present in the pheromone gland, but may show qualitative and quantitative differences from the naturally emitted semiochemical blend. Ratios are a fundamental aspect of many semiochemical systems. For a number of aphid species, the sexual females (oviparae) attract winged males by releasing a sex pheromone from porous plaques on their hind tibiae. Using solvent extraction of the female tibiae, it was demonstrated that the sex pheromone of the vetch aphid, *Megoura viciae*, comprises a synergistic mixture of the monoterpenoids (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. However, with an air entrainment technique, collecting volatiles from the air above calling oviparous *M. viciae*, it was found that the nepetalactone was released in significantly higher concentrations than was found in the leg extracts (**Figure 2**). Whilst only a small amount of the nepetalactone (1–2 ng per aphid) was obtained from the leg extract, the amount produced by calling females was 200 ng per aphid.

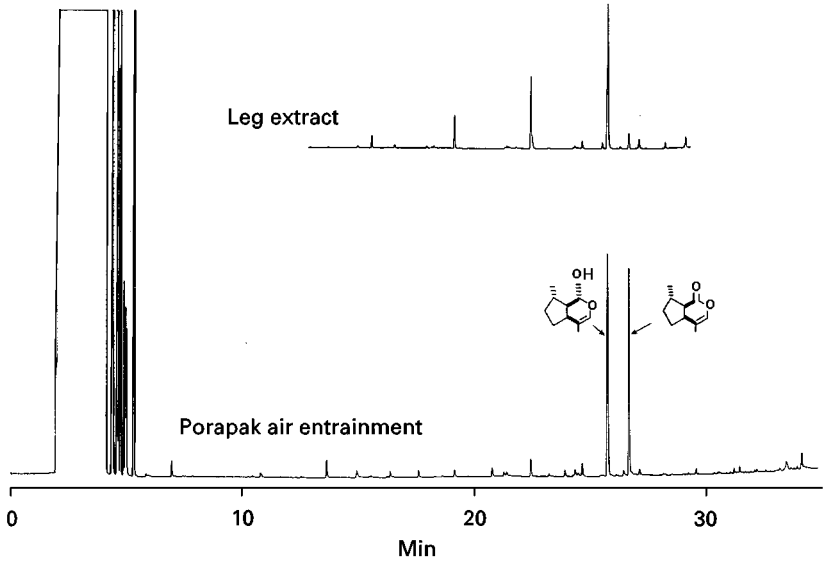


Figure 2 GC of pheromone components produced by sexual female aphids (oviparae): amounts obtained by air entrainment and (inset) solvent extraction of excised hind tibiae.

These findings suggested that, during calling, production of nepetalactol is continuous and that a proportion of the nepetalactol is sequentially oxidized to nepetalactone. These two compounds, in different ratios, were shown to be the main components comprising the sex pheromones of many aphid species.

The problems associated with solvent extraction can be overcome by using dynamic entrainment systems, where the organism emitting the semiochemicals is contained in a glass chamber through which is passed a stream of highly purified air. The volatiles produced are swept from the system and collected in a trap, usually a porous polymer, activated charcoal or a cryogenic trapping system. They can then be recovered from the trap by either solvent or thermal desorption. The advantages of using such a system are that not only are the semiochemicals isolated in the proportions emitted by the organism, but also the extracts obtained are free from contaminants associated with straight solvent extraction and are thus directly amenable to analysis by GC. In addition, since the sampling is not destructive, it can be used to investigate the time course of semiochemical production.

Solid-phase microextraction (SPME) is a relatively new isolation technique that has been developed for the extraction and concentration of a wide range of volatile and semivolatile organic compounds from various matrices such as air, water and soil. The technique can be utilized for direct immersion into liquid samples and for sampling the headspace of liquid and solid samples. SPME uses a polymer-coated fibre to absorb chemicals from the matrices, relying on a three-phase equilibrium between the sample, its vapour and the fibre. Samples are desorbed from the SPME fibre by thermal desorption in the GC injector. A number of fibres have been produced using different types of coating, e.g. polydimethylsiloxane (100 μm) for volatile chemicals, carbowax/DVB for alcohols and other polar compounds and polyacrylate for semivolatile compounds. Although the technique is still in a developmental stage, its ability to collect insect pheromones has already been demonstrated. However, SPME is not without its drawbacks, particularly when used for quantification, since adsorption on to the fibre is related to the chemical properties of the compound, with some compounds being adsorbed more readily than others. The time needed for a compound to equilibrate with the fibre is related to the structure of the compound, and experimental conditions such as temperature and humidity can affect the adsorptive capacity of the fibre. Despite these limitations, SPME has several major advantages in that it is rapid, nondestructive and allows sequential samples to be taken.

Sample Introduction

Analysis of complex samples places a great demand on the inlet (injection) system of the GC. The injection techniques used in capillary GC are split/splitless, direct, cold on-column and temperature-programmed vaporization. Considerable emphasis has been placed upon developing improved sample introduction techniques, minimizing sample de-

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composition or isomerization, and the delivery of all components of the sample into the column in the same proportions as in the original mixture. Cold on-column injection techniques, where the sample is introduced directly on to the capillary column, can largely overcome these problems. However, this imposes considerable constraints on the initial isolation techniques, since it is essential that the sample is free from high molecular weight contaminants. Another versatile and popular technique is programmed-temperature vaporization, where the sample is introduced into a cold injector port followed by a rapid temperature rise, thus achieving vaporization.

The use of solvents can present problems for the chemical ecologist. Even highly purified solvents, when analysed on a modern high efficiency capillary GC system, show considerable levels of impurities. In addition, very volatile components can be masked by the solvent peak. Hence, in many cases, especially when working at extreme sensitvities, it is preferable to work with solvent-free systems and a number of injection systems for this have been developed. Although many were initially designed for use with packed columns, there is no fundamental difficulty in adapting them for use with capillary GC. In one of these solid sample injection systems, the material to be analysed is sealed inside a glass capillary, which is then placed inside the heated injector for a few minutes to allow the sample to volatilize, or for a biological sample to heat through. The tube is then crushed with a plunger and the volatile material is swept directly on to the column by the carrier gas. The advantages of this technique are simplicity of operation, reduced chance of contamination and no dilution. Even highly volatile compounds such as methanol and acetaldehyde can be recognized and quantified.

Thermal desorption provides another method of introducing samples on to the GC column without the use of solvents. Volatiles collected by dynamic air entrainment systems on to porous polymer traps can be removed either by solvent elution, or by thermal desorption in the GC injector. In the latter instance, all of the sample is introduced on to the GC column, thus increasing sensitivity and allowing analysis of compounds with short retention times. However, thermal desorption is not without its own drawbacks, particularly the thermal instability of some compounds.

GC Columns

Modern capillary GC columns have great resolving power and offer a high speed of analysis, greater sensitivity and the capacity to elute a greater range of components, providing that the molecules of solute to be analysed are thermally stable, inert in terms of reacting with the capillary column and have sufficient volatility. As with all GC analyses, the most important parameters to be considered in selecting the best column are the stationary phase, internal diameter, film thickness and length. The stationary phase used in the column has the greatest effect in separation. Depending on the chemical properties of the compound, some compounds separate better on some stationary phases than on others. However, since semiochemical analyses are usually performed on complex mixtures comprising compounds with a wide range of boiling points and functionalities, the choice of column is frequently a compromise. This is not usually a problem since the high efficiency of modern capillary columns ensures that most analyses can be performed on a limited range of phases. Indeed, the stationary phases most commonly used in pheromone research are nonpolar (e.g. 100% polydimethylsiloxane), polar (e.g. polyethylene glycol) or medium polarity (e.g. poly[diphenyldimethylsiloxane] copolymer). GC columns with polar phases are invaluable for the separation of many pheromones, particularly fatty acid-derived lepidopterous pheromones.

Animal – and particularly insect – olfactory receptor systems are highly specialized and are frequently able to distinguish between enantiomers. Thus, in human olfaction, the two enantiomers of carvone (*p*-mentha-6,8-dien-2-one), a chiral cyclic ketone with one asymmetric carbon atom, have very different odours. One has the odour of caraway [(*R*)-carvone] and the other of spearmint [(*S*)-carvone]. Chirality plays a key role in insect chemical ecology and biological activity is often dependent on the enantiomeric composition of a chiral compound, and situations exist where the 'non-natural' enantiomer is inactive or, more problematically, may even elicit a repellent response. Japonilure, the female-produced sex pheromone of the Japanese beetle, *Popillia japonica*, comprises (*R*)-(*Z*)-5-(1-decenyl)dihydro-2-(3*H*)-furanone (Figure 1, structure **2**) and males require high enantiomeric purity of the (*R*)-isomer to be attracted to the sex pheromone. However, males also possess receptors for the (*S*)-isomer which, when perceived simultaneously with the (*R*)-enantiomer, has an inhibitory effect, and only a few per cent of the wrong enantiomer can inhibit the response to the natural pheromone. Geographical variation in the production of, and response to, the enantiomers of the aggregation pheromone ipsdienol (Figure 1, structure **3**) has been demonstrated for the pine engraver, *Ips pini*, in eastern and western populations in the USA. Thus, determination of the chirality of such

pheromones is of paramount importance in chemical ecology. However, until recently, such determinations have been limited to the sampling of large numbers of insects for determination of optical rotation by conventional methods, e.g. Fourier transform nuclear magnetic resonance.

GC techniques are preferred for enantiomer composition studies since they are very sensitive, require less sophisticated instrumentation and can be applied even to small amounts of impure biological samples. Chiral GC phases, where optical resolution is achieved through reversible diastereomeric association between the chiral environment and the solute enantiomer by means of molecular interactions such as hydrogen bonding, inclusion phenomena, transition metals or charge transfer interactions, have been used for the determination of the enantiomeric composition of pheromones. One class of widely distributed pheromone components are the spiroketals isolated from various insects such as bees, wasps and beetles. The first such pheromone to be identified was chalcogran, or 2-ethyl-1,6-dioxaspiro(4,4)nonane, the pheromone of the beetle *Pityogenes chalcographus*, which was resolved by complexation GC with a capillary column coated with nickel(II)-bis(6-heptafluorobutyryl)-(*R*)-pulegonate.

Although some chiral columns, e.g. the cyclodextrins, are now available commercially, they are relatively expensive and column performance can deteriorate quite rapidly. A convenient and attractive alternative to the direct separation of enantiomers is the GC separation of a diastereomeric derivative formed with an optically pure derivatizing agent, on achiral stationary phases which are less expensive and more generally available. A number of derivatives are available, particularly for alcohols, of which some of the most effective are the *N*-trifluoroacetyl (*S*)-alanyl esters, *N*-trifluoroacetyl-(*S*)-prolyl esters and (*R*)-*trans*-chrysanthemoyl esters. Using the latter derivatives, it is possible to obtain baseline separation of the chrysanthemate esters of 3-octanol on a simple OV-1 column. With highly purified reagents, it is possible to convert chiral alcohols and latent alcohols into separable diastereomers, enabling chiral determinations to be made on unpurified extracts of individual insects. Information on variation of pheromone chirality may have profound implications, both for population studies and for investigating the impact of pest management programmes.

Detectors

There are many types of detectors used in GC which have different degrees of selectivity. Of these, the flame ionization detector (FID) gives a response for almost all carbon-containing compounds. Allied to this, it is rugged and relatively insensitive to operating variables and has a low dead volume and an extremely wide linear range of response. These properties have ensured that it is by far the most commonly used of all detectors in chemical ecology. Another widely used detector is the mass spectrometer and, indeed, advances in understanding the subject have been achieved largely as a result of developments in this field.

GS–mass spectrometry (GC–MS) is now the method of choice for initial semiochemical identification and, in many instances, is the only technique available and able to provide structural information at the very low sample levels usually encountered in such studies. Mass spectrometers can range from sophisticated instruments with a variety of soft ionization techniques, very high resolving power and extended mass ranges, to small bench-top instruments with limited mass ranges and other capabilities. GC–MS can also be used in quantification and selected ion monitoring (SIM) is a widely accepted quantitative technique based on monitoring the ion abundance of selected m/z values. The sensitivity of SIM for a given compound may be 1000 times as great when monitoring selected ions as when scanning the complete mass spectrum.

In addition to the universal detectors, a number of selective detectors are used. Typically, the extracts obtained from insect or plant sources may contain several hundred components, the vast majority of which are not behaviourally active. The location of the active components in such cases presents a considerable problem for chemical ecologists. This was often attempted in the past by preparative GC of the sample, followed by testing of each of the fractions in a laboratory behavioural assay. By repetitive fractionation and bioassay, it was hoped that fractions containing a single active compound could be obtained for subsequent identification studies. However, this procedure was exceedingly time-consuming and, since many pheromone systems comprise more than one compound, often fruitless. Thus, considerable efforts have been directed towards the development of selective detectors that can be coupled directly to the GC to allow rapid location of biologically active components in complex natural product extracts.

Insects perceive volatile semiochemicals via olfactory receptors, usually located on the antenna. These olfactory cells are extremely sensitive and are tuned to the detection of semiochemicals involved in the insect's chemical ecology. When stimulated, these receptors transform the chemical signal into a series of electrical events which are then passed directly to the

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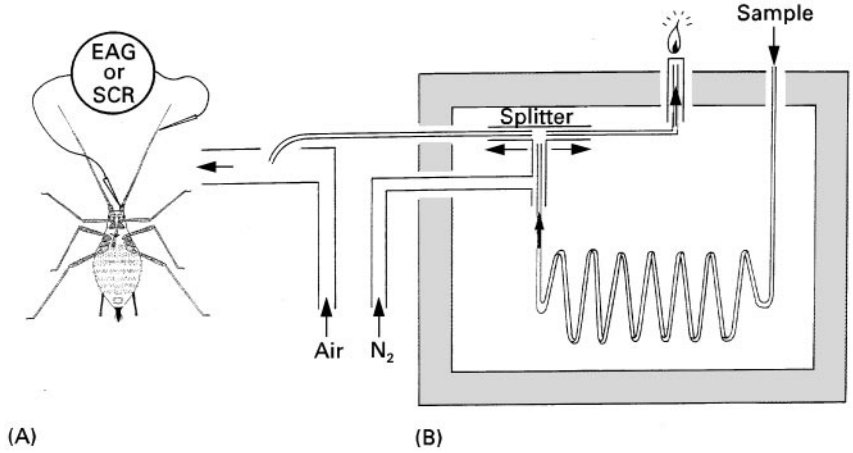

Figure 3 The coupled GC–electrophysiology system. (A) Antennal detector; (B) GC detector. EAG, electroantennogram; SCR, single-cell response.

insect's central nervous system. By placing electrodes in the antenna, it is possible to record these electrical events. At the simplest level, this can be achieved by excising the antenna and suspending it between two electrodes connected to an amplifier system. The so-called electroantennogram, obtained from the antenna on stimulation with an appropriate semiochemical, can then be displayed on an oscilloscope, chart recorder or computer screen. At a more sophisticated level, the responses from the individual olfactory neurons on the antenna can also be recorded (single-cell responses). These electrophysiological preparations have a relatively long lifetime, ranging from tens of minutes to several hours, and thus offer considerable potential for the development of highly specific GC detectors. By linking these preparations to high resolution gas chromatography, i.e. splitting the effluent from the GC column and presenting it simultaneously to the FID of the GC and to the antennal preparation (**Figure 3**), it is possible to locate biologically active compounds even within highly complex extracts. Since modern high resolution GC columns typically have peak widths of only a few seconds, considerable care must be taken to ensure

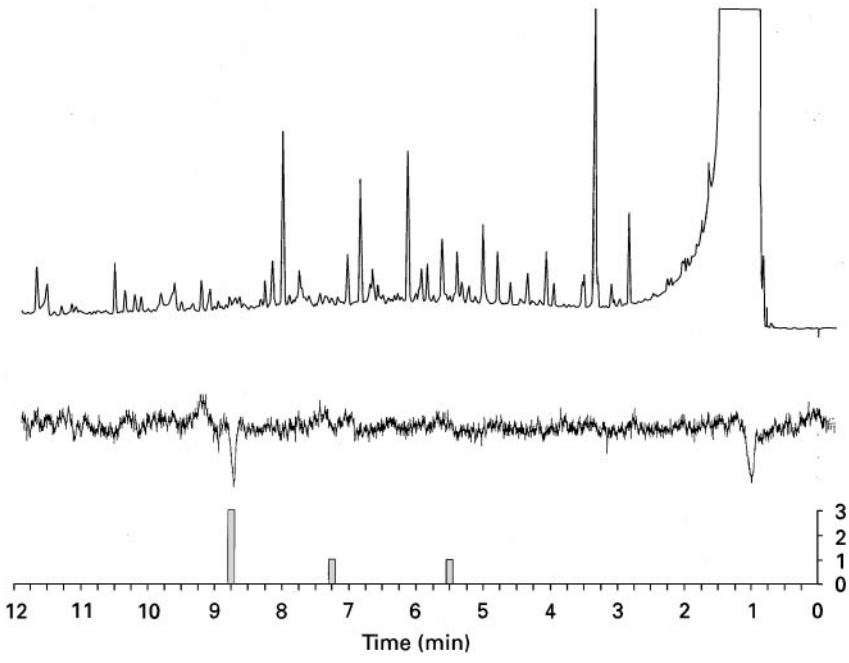

Figure 4 Coupled GC–electroantennogram and GC–behavioural assay with males of the aphid parasitoid *Praon volucre*. Upper trace: GC of volatiles from virgin females; middle trace: electroantennogram response from one male; lower columns: behavioural responses (number of males, out of five tested, showing wing-fanning activity).

that components eluting from the column arrive simultaneously at the two detectors.

Coupled GC–electrophysiological techniques have provided a powerful tool in the chemical ecologist's armoury, enabling accurate targeting of specific peaks for subsequent identification by GC–MS. Indeed, the accuracy of these systems is such that, even where no GC–MS peak is observed, it may still be possible to extract sufficient information from the MS data system to allow a tentative identification to be achieved. By adding marker peaks to the sample that elute either side of the electrophysiologically active component, or by using other compounds present in the original sample which show up on the GC–MS trace, the researcher can pinpoint accurately the region in the GC–MS chromatogram where the electrophysiologically active peak should elute. Manual interrogation of the data system can then identify specific ions that peak at the expected scan numbers. Such identifications, on picograms of material, would not be possible without the accurate information on where to look in the chromatogram that is provided by coupled GC–electrophysiological studies.

Although electrophysiological activity of a compound suggests that it is involved in some aspect of the insect's chemical ecology, it gives no indication of the behavioural role. Confirmation of the behavioural relevance of a particular component in the chromato-

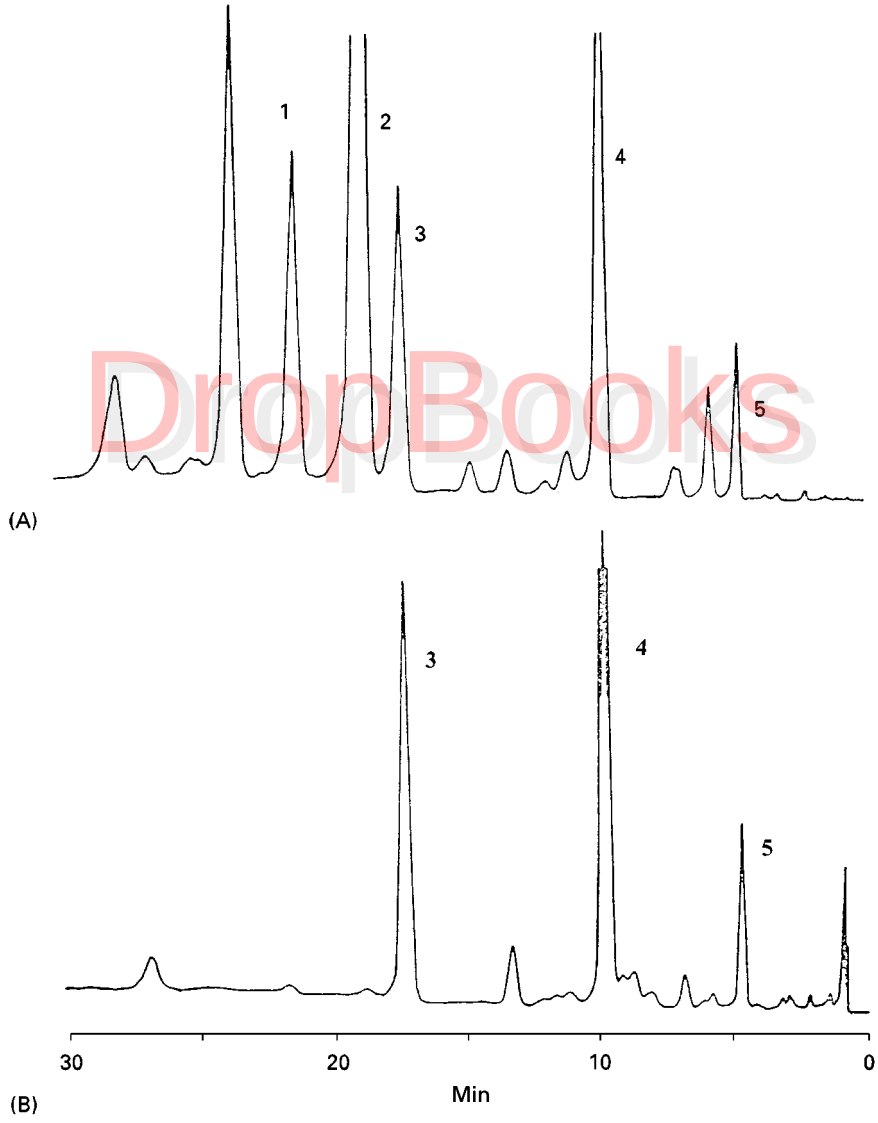

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Figure 5 Reaction chromatography. Bromination of alkenes. (A) Gas chromatogram of the Dufour gland contents of the ant *Myrmica rubra*, obtained using solid sampling technique with a packed column (2.75 m × 4 mm of 10% polyethylene glycol adipate on gas chrom M.). (B) A gland from another worker treated with bromine during injection with the solid sampling technique. 1, α-Farnesene; 2, 8-heptadecene; 3, heptadecane; 4, pentadecane; 5, tridecane.

gram can be obtained by linking the GC to a simple behavioural assay, for example the wing-fanning response elicited from male aphid parasitoids by the female sex pheromone (**Figure 4**). By monitoring the responses of individuals or groups of insects in the bioassay chamber as they are exposed to the effluent from the GC, it is possible to locate, quite accurately, the elution time of the semiochemical. This technique was widely used in the 1970s, particularly in studies on lepidopterous sex pheromones. More recently, this approach has been used to investigate the role of learning in mixture recognition by foraging honeybees.

Reaction Gas Chromatography

GC–MS is the main identification tool for chemical ecologists, but frequently does not provide sufficient information for a full characterization of the compounds of interest. Thus, in terms of lepidopterous sex pheromones, which usually comprise long chain fatty acid derivatives with varying degrees of unsaturation, MS frequently cannot locate the positions of double bonds, nor distinguish between (*Z*)- and (*E*)-isomers. Microscale reactions conducted before chromatography, or even on-column, can be used to provide information about the class of compound and its functional group, or even to convert them into more stable derivatives. It is possible to carry out a surprising number of reactions on nanogram quantities of material where the reaction is reproducible, quantitative and gives simple products. Various methods have been described for hydrogenation, ozonolysis, epoxidation, reduction, hydrolysis and esterification on nanogram sample levels, or even the use of subtraction loops or specific reactions to remove particular classes of compounds from the mixture (**Figure 5**).

Conclusions

For the chemical ecologist, GC is not just a technique that enables high resolution and separation of complex natural product extracts. It can also provide considerable structural information. At the simplest level, noting the retention times of compounds of interest on polar and nonpolar stationary phases can give information on the molecular mass and polarity of a compound. However, when appropriate microscale reactions are included in the repertoire, the GC can prove to be a considerable aid in the identification of semiochemicals. When combined with MS, the availability of structural information is increased considerably.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry; Headspace Gas Chromatography. **III/Chiral Separations:** Gas Chromatography.

Further Reading

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Thin-Layer (Planar) Chromatography

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Thin-layer chromatography (TLC) in the study of pheromones is more a subject of potentials than of wide application. Its use, some examples of its application in general and specific problems, and some sources where the reader can find procedures to follow are described here.

There is a constant argument between those who advocate simpler methods and techniques (often the